Role of Cytochromes P450 1A1/2 in Detoxication and Activation of Carcinogenic Aristolochic Acid I: Studies with the Hepatic NADPH:Cytochrome P450 Reductase Null (HRN) Mouse Model

Kateřina Levová*, Michaela Moserová*, Věra Kotrbová*, Miroslav Šulc*, Colin J. Henderson[†], C. Roland Wolf[†], David H. Phillips[‡], Eva Frei[§], Heinz H. Schmeiser[¶], Jaroslav Mareš[‖1], Volker M. Arlt[‡], Marie Stiborová*[1]

**Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic*

[†]*Cancer Research UK Molecular Pharmacology Unit, Biomedical Research Centre, Dundee DD1 9SY, United Kingdom*

[‡]*Section of Molecular Carcinogenesis, Institute of Cancer Research, Brookes Lawley Building, Sutton, Surrey SM2 5NG, UK*

[§]*Division of Preventive Oncology, National Center for Tumour Diseases, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*

[¶]*Research Group Genetic Alterations in Carcinogenesis, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*

[‖]*Institute of Biology and Medical Genetics, 2nd Medical School, Charles University and University Hospital Motol, V Úvalu 84, 150 06 Prague 5, Czech Republic*

[1]To whom correspondence should be addressed at *Department of Biochemistry, Faculty of Science, Charles University Prague, Albertov 2030, 12840 Prague 2, Czech Republic*, Fax: +420-2-21951283. E-mail: stiborov@natur.cuni.cz or at *Institute of Biology and Medical Genetics, 2nd Medical School, Charles University and University Hospital Motol, V Úvalu 84, 150 06 Prague 5, Czech Republic,* Fax: +420 224433520. E-mail: jaroslav.mares@lfmotol.cuni.cz

ABSTRACT

Aristolochic acid (AA) causes aristolochic acid nephropathy, Balkan endemic nephropathy and their urothelial malignancies. To identify enzymes involved in the metabolism of aristolochic acid I (AAI), the major toxic component of AA we used HRN [Hepatic Cytochrome P450 (Cyp) Reductase Null] mice, in which NADPH:Cyp oxidoreductase (Por) is deleted in hepatocytes. AAI was demethylated by hepatic Cyps *in vitro* to 8-hydroxyaristolochic acid I (AAIa), indicating that less AAI is distributed to extra-hepatic organs in wild-type (WT) mice. Indeed, AAI-DNA-adduct levels were significantly higher in organs of HRN mice, having low hepatic AAI demethylation capacity, than in WT mice. Absence of AAI demethylation in HRN mouse liver was confirmed *in vitro*; hepatic microsomes from WT, but not from HRN mice, oxidized AAI to AAIa. To define the role of hepatic Cyps in AAI demethylation, modulation of AAIa formation by CYP inducers was investigated. We conclude that AAI demethylation is attributable mainly to Cyp1a1/2. The higher AAI-DNA adduct levels in HRN than WT mice were the result of the lack of hepatic AAI demethylation concomitant with a higher activity of cytosolic NAD(P)H:quinone oxidoreductase (Nqo1), which activates AAI. Mouse hepatic Cyp1a1/2 also activated AAI to DNA adducts under hypoxic conditions *in vitro*, but in renal microsomes, Por and Cyp3a are more important than Cyp1a for AAI-DNA adduct formation. We propose that AAI activation and detoxication in mice are dictated mainly by AAI binding affinity to Cyp1a1/2 or Nqo1, by their turnover and by the balance between oxidation and reduction of AAI by Cyp1a.

Key Words: aristolochic acid; cytochrome P450; metabolism; DNA adducts; ^{32}P-postlabeling

The cytochrome P450 (CYP) superfamily consists of a large number of haem-containing mono-oxygenases that play a pivotal role in the metabolism of many drugs and carcinogens (Nebert and Dalton, 2006). Vital information on CYP regulation and function has been obtained by *in-vitro* studies, however, for extrapolation from *in vitro* data to *in vivo* pharmacokinetics additional factors such as route of administration, absorption, renal clearance and tissue-specific CYP expression need to be considered (Nebert, 2006). Gene knock-out and transgenic mice have been developed to study the role of specific enzymes in drug metabolism (Henderson et al., 2003b). Although knock-out mouse models have yielded important data on the effect of single Cyp enzymes on the metabolism of drugs and chemical carcinogens (Buters et al., 1999; Kimura et al., 2003; Tsuneoka et al., 2003; Uno et al., 2004), the functional redundancy inevitably found in the large CYP family of isoenzymes make it difficult to determine the role of CYPs as a whole in the metabolism of xenobiotics (Henderson et al., 2006). To overcome these limitations a mouse line, HRN (Hepatic Cytochrome P450 Reductase Null), has been developed in which Cyp oxidoreductase (Por), the important electron donor to Cyps, is deleted specifically in hepatocytes, resulting in the loss of essentially all hepatic Cyp function (Henderson et al., 2003a). The HRN mouse model has been used successfully to establish the role of hepatic *versus* extra-hepatic Cyp-mediated xenobiotic metabolism and disposition (Arlt et al., 2005; 2006; 2008; Pass et al., 2005; Stiborová et al., 2008a).

The herbal drug aristolochic acid (AA) derived from Aristolochia species has been shown to be the cause of so-called Chinese herbs nephropathy (CHN), now termed aristolochic acid nephropathy (AAN) (Debelle et al., 2008; Schmeiser et al., 2009). The plant extract AA is a mixture of structurally related nitrophenanthrene carboxylic acids, the major components being aristolochic acid I (AAI; Fig. 1) and aristolochic acid II. AAN is a rapidly progressive renal fibrosis that was observed initially in a group of Belgian women who had ingested weight loss pills containing *Aristolochia fangchi* (Vanherweghem et al., 1993). Within a few years of taking the pills, AAN patients also developed a high risk of upper urothelial tract carcinoma (about 50%) and,

subsequently, bladder urothelial carcinoma (Nortier et al., 2000; Lemy et al., 2008). Subsequently, similar cases have been reported elsewhere in Europe and Asia (Lord et al., 2001; Debelle et al., 2008; Lai et al., 2010). More recently, exposure to AA has been linked to Balkan endemic nephropathy (BEN) and its associated urothelial cancer (Arlt et al., 2007; Grollman et al., 2007; Nedelko et al., 2009). This nephropathy is endemic in certain rural areas of Serbia, Bosnia, Croatia, Bulgaria and Romania. Exposure to AA was demonstrated by the identification of specific AA-DNA adducts in urothelial tissue of AAN and BEN patients (Schmeiser et al., 1996; Bieler et al., 1997; Nortier et al., 2000; Lord et al., 2001; Arlt et al., 2002; Grollman et al., 2007). The most abundant DNA adduct detected in patients exposed to AA is 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI), which leads to characteristic AT→TA transversion mutations. Such AT→TA mutations have been observed in the *TP53* tumor suppressor gene in tumors from AAN and BEN patients (Lord et al., 2004; Arlt et al., 2007; Grollman et al., 2007; Nedelko et al., 2009), indicating the probable molecular mechanism of AA carcinogenesis in humans (Simoes et al., 2008; Arlt et al., 2011). As a consequence, AA was recently classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) (Grosse et al., 2009).

In common with other nitroaromatics the major activation pathway for AA is nitroreduction catalyzed by both cytosolic and microsomal enzymes, cytosolic NAD(P)H:quinone oxidoreductase (NQO1) being the most efficient (Stiborová et al., 2002a; 2003a; 2008b; 2011) (Fig. 1). The activation of AAI in human hepatic microsomes is mediated by CYP1A2 and, to a lesser extent by CYP1A1; POR also plays a minor role (Stiborová et al., 2001b; 2001c; 2005a; 2005c). Prostaglandin H synthase (cyclooxygenase, COX) in human renal microsomes has also been shown to activate AAI (Stiborová et al., 2001a; 2005a). While the enzymes catalyzing the reductive activation of AAI leading to covalent DNA adducts have been widely investigated, those participating in its detoxication have not been extensively studied so far. Several studies have indicated that induction of Cyp1a (e.g. by 3-methylcholanthrene and ß-naphthoflavone) protect

mice from AAI-induced acute renal injury (Xue et al., 2008; Xiao et al., 2008; 2009). One detoxication metabolite identified is 8-hydroxy-aristolochic acid I (aristolochic acid Ia, AAIa; Fig. 1) that is formed after demethylation of AAI and is, in turn, subject to conjugation, forming glucuronide or sulfate esters (Krumbiegel et al., 1987; Chan et al., 2007; Shibutani et al., 2010). Human CYP1A1 and -1A2 can demethylate AAI to AAIa *in vitro* (Šístková et al., 2008; Rosenquist et al., 2010) and Cyp1a2 in mice appears to mediate this reaction *in vivo* (Rosenquist et al., 2010). Nevertheless, because CYP1A1/2 also activate AAI in human and rat livers (Stiborová et al., 2001a; 2005a; 2005c; 2008b) detailed knowledge of the catalytic specificities of CYP1A and other CYP enzymes in the detoxication and activation of AAI *in vitro* and *in vivo* is still lacking.

The aim of the present study was to evaluate the CYP-mediated oxidative detoxication of AAI. We have used the HRN mouse model to examine the hepatic Cyp-dependent metabolism of AAI. DNA adduct formation *in vivo* and *in vitro* was measured by ^{32}P-postlabelling. In addition, the formation of AAIa by human, rat and rabbit hepatic microsomes, and by rat recombinant CYPs was determined by high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Animal treatment

HRN ($Por^{lox/lox}$ + Cre^{ALB}) mice on a C57BL/6 background (CXR Bioscience Ltd, Dundee, UK) used in this study were derived as described previously (Henderson et al., 2003a). Mice homozygous for loxP sites at the *Por* locus ($Por^{lox/lox}$) were used as wild-type (WT). Groups of male HRN and WT mice (3 months old; 25-30 g; n = 3/group) were treated orally with a single dose of 10 or 50 mg/kg body weight (bw) of AAI [as sodium salt isolated from the natural mixture of AA (38% AAI, 58% AAII; Sigma Chemical Co, St Louis, MO, USA) by preparative HPLC (Schmeiser et al., 1984)] at a concentration of 1 or 5 mg/ml, respectively. Control mice received solvent, water, only. Animals were killed 24 h after treatment. Liver, lung, kidney, spleen, colon, small intestine and bladder were removed, snap frozen and stored at −80°C until analysis. All procedures were carried out under the Animal (Scientific Procedures) Act (1986) in accordance with UK law, and following local ethical review.

DNA adduct analysis by ^{32}P-postlabeling

DNA from tissues was isolated by standard phenol/chloroform extraction. ^{32}P-Postlabelling analysis (Phillips and Arlt, 2007) using the nuclease P1 enrichment version, and thin layer chromatography (TLC) and HPLC were performed as described (Schmeiser et al., 1996; Bieler et al., 1997). TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Schmeiser et al., 1996; Bieler et al., 1997). Results were expressed as DNA adducts/10^8 nucleotides.

Preparation of microsomes and cytosols

Hepatic and renal microsomes and cytosols from HRN and WT mice were isolated as described (Stiborová et al., 2003b; 2005a). Hepatic and renal microsomes and cytosols from HRN and WT

mice pre-treated (i.p.) with 125 mg/kg bw benzo[*a*]pyrene (BaP) daily for 5 days were obtained from a previous study (Arlt et al., 2008). Pooled microsomal and cytosolic fractions were used for further analysis. Male human (pooled sample; cat. no. 452172) and human female hepatic microsomes (pooled sample; cat. no. 452183) were from Gentest Corp. (Woburn, MI, USA). Microsomes were prepared from livers of ten untreated Wistar rats and three Chinchilla rabbits by the procedure described previously (Stiborová et al., 1995; 2002b). Microsomes were also prepared from livers of groups of ten Wistar male rats pre-treated with Sudan I, phenobarbital (PB), ethanol (EtOH) or pregnenolone-16α-carbonitrile (PCN) as described previously (Stiborová et al., 2002b; 2003b).

Microsomal incubations used for AAI-DNA adduct analysis

The deaerated and argon-purged incubation mixtures, in a final volume of 750 μL, consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg mouse hepatic or renal microsomal protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI as described previously (Stiborova et al. 2005a). Incubations with microsomes were carried out at 37°C for 60 min and AAI-derived DNA adduct formation was found to be linear up to 2 hr (Stiborová et al., 2005a).

Microsomal incubations used for AAI demethylation

Incubation mixtures, in a final volume of 250 μL, consisted of 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg human, rat, mouse or rabbit hepatic or mouse renal microsomal protein and 10 μM AAI. Incubations with microsomes were carried out at 37°C for 20 min and AAI oxidation (demethylation) to AAIa was linear up to 25 min. Control incubations were carried out (*i*) without microsomes, (*ii*) without NADPH or (*iii*) without AAI. Supersomes™, microsomes isolated from insect cells transfected with baculovirus constructs containing cDNA of single rat CYPs (CYP1A1, CYP1A2, CYP2A1, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2C13,

CYP2D1, CYP2D2, CYP2E1, CYP3A1, and CYP3A2), and expressing POR and/or cytochrome b_5 were obtained from Gentest Corp and tested for their efficiencies to oxidize AAI. Incubation mixtures, in a final volume of 250 µL, consisted of 100 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM $MgCl_2$, 10 mM D-glucose 6-phosphate, 1 U/mL D-glucose 6-phosphate dehydrogenase, to generate NADPH, 50 nM CYPs in Supersomes™ and 10 µM AAI. Supersomes containing POR alone were used for control.

Determination of AAIa by HPLC

AAI and its metabolites (*i.e.* AAIa) were extracted from incubation mixtures twice into ethyl acetate (2 × 1 mL), the extracts were evaporated to dryness and the residues redissolved in 30 µL of methanol and subjected to reverse-phase HPLC. HPLC was performed with a reversed phase column (Nucleosil 100-5 C_{18}, 25 × 4.0 mm, 5 mm; Macherey-Nagel) preceeded by a C-18 guard column, using a linear gradient of acetonitrile (20-60% acetonitrile in 55 min) in 100 mM triethylamonium acetate with a flow rate of 0.6 mL/min. HPLC was carried out with a Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometer detector set at 254 nm, and peaks were integrated with CHROMELEON™ 6.01 integrator. A product of AAI metabolim and AAI itself eluted with retention times (r.t.) of 28.3 and 36 min, respectively. The product eluting at 28.3 min was identified as AAIa using mass-spectroscopy analyses. Mass spectra were measured on MALDI-TOF/TOF ultraFLEX III mass spectrometers (Bruker-Daltonics, Bremen, Germany). Positive spectra were calibrated externally using the monoisotopic $[M+H]^+$ ions of PepMixII calibrant (Bruker-Daltonics, Bremen) or matrix peaks. A 10 mg/mL solution of α-cyano-4-hydroxy-cinnamic acid, or 50 mg/mL solution of 2,5-dihydrobenzoic acid in 50% MeCN/0.1% TFA were used as MALDI matrix. A 0.5 µL sample dissolved in MeCN was directly mixed with 0.5 µL of the matrix solution and allowed to dry at ambient temperature on the target. For NALDI experiments, a

1−μL sample dissolved in MeCN was allowed to dry at ambient temperature on NALDI target. The MALDI- or NALDI-TOF positive spectra were collected in reflector mode.

Cytosolic incubations used for AAI-DNA adduct analysis

The deaerated and argon-purged incubation mixtures, in a final volume of 750 μL, consisted of 50 mM Tris-HCl buffer (pH 7.4), containing 0.2% Tween 20, 1 mM NADPH, 1 mg murine hepatic or renal cytosolic protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI as described previously (Stiborova et al., 2003a). Incubations with human cytosols were carried out at 37°C for 60 min and AAI-derived DNA adduct formation was found to be linear up to 2 hr (Stiborová et al., 2003a).

Inhibition studies

α-Naphthoflavone (α-NF), which inhibits Cyp1a1 and -1a2 (Stiborová et al., 2001b; 2005c); ellipticine (E), which competes with Cyp1a1 substrates, thus inhibiting Cyp1a1-mediated oxidation of other substrates (Stiborová et al., 2003b; 2004); furafylline (FF), which inhibits Cyp1a2 (Stiborová et al., 2001b); ketoconazole (KC), which inhibits Cyp3a4 (Stiborová et al., 2001b); and α-lipoic acid (α-LA), which inhibits Por (Arlt et al., 2003; Stiborová et al., 2005b) were used to inhibit the activation of AAI in murine microsomes. Dicoumarol was used to inhibit the activation of AAI by Nqo1 in mouse hepatic cytosols. The inhibition studies were performed as described (Stiborová et al., 2001b ; 2003a; Arlt et al., 2005).

Determination of NQO1activity and protein levels by Western blotting

NQO1 antibodies were prepared as described previously (Stiborová et al., 2002b; 2005b; 2006). Immunoquantitation of cytosolic NQO1 was carried out by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Stiborová et al., 2006). Human recombinant NQO1 (Sigma)

was used to identify the band Nqo1 in murine cytosols. NQO1 activity was measured essentially as described by Ernster (1976), but the method was improved by addition of cytochrome *c* (Mizerovská et al., 2011).

RESULTS

DNA adduct formation by AAI in HRN mice

In order to evaluate the role of hepatic Por-dependent Cyp enzymes in the detoxication of AAI *in vivo* we treated HRN and WT mice with a single dose of 10 or 50 mg/kg bw of AAI by oral gavage. The formation of AAI-DNA adducts in various organs (liver, lung, kidney, spleen, colon, small intestine and urinary bladder) was determined by ^{32}P-postlabeling. Essentially the same DNA adduct pattern as that found *in vivo* in humans (Schmeiser et al., 1996; Bieler et al., 1997) and rats (Schmeiser, et al., 1988; Stiborová et al., 1994) and in incubations using rat and human hepatic microsomes (Stiborová et al., 2001b, 2005a) and human hepatic and renal cytosols (Stiborová et al., 2003a), was observed in all tissues analyzed (see Figure 1 inset for kidney of mice treated with 50 mg/kg bw AAI). Adducts were indentified to be 3´,5´-bisphospho-7-(deoxyguanosin-N^2-yl)-aristolactam I (dG-AAI, spot 1), 3´,5´-bisphospho-7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI, spot 2) and 3´,5´-bisphospho-7-(deoxyadenosin-N^6-yl)-aristolactam II (dA-AAII, spot 3) (Pfau et al., 1990; Stiborová et al., 1994; Schmeiser et al., 1996; Fig. 1). The identities of the individual AAI-derived DNA adducts were confirmed by cochromatographic analysis on TLC and HPLC as described previously (Schmeiser et al., 1996) (data not shown). No DNA adducts were observed in DNA isolated from the tissues of control animals (data not shown).

AAI-DNA adduct formation was concentration-dependent and organ-specific (Fig. 2 and Supplementary Table S1). Adduct levels in WT mice were highest in the small intestine, followed by liver, colon, bladder, lung, spleen and kidney, where adducts were only observed after 50 mg/kg (Fig. 2B). In HRN mice treated with either AAI dose higher levels of AAI-DNA adducts were found than in WT mice in all organs analyzed, with the highest levels and largest differences in kidney (Fig. 2B). The latter finding indicates that the lack of Por-dependent Cyp-mediated detoxication of AAI in liver of HRN mice increased the effective concentration of AAI in this organ and in extra-hepatic tissues.

Human, mouse, rat and rabbit hepatic microsomes oxidize AAI to AAIa

In-vitro experiments were performed to further examine the role of the mouse hepatic Cyps in the detoxication of AAI. Microsomes were isolated from livers of HRN and WT mice, either untreated or pretreated with BaP (*i.e.* HRN-BaP and WT-BaP mice, Arlt et al., 2008). For comparison, human, rabbit and rat hepatic microsomes were utilized.

Hepatic microsomes of WT, WT-BaP and HRN-BaP mice were capable of metabolizing AAI to one metabolite detectable by HPLC analysis (see peak with r.t. of 28.3 min in Figure 3 for microsomes of WT mice). MS (NALDI- and MALDI-TOF-TOF, Fig. 4) was used to identify the structure of this metabolite. Negative NALDI-TOF-TOF MS detected a peak at *m/z* 325.803 (Fig. 4A), representing the molecular ion $[M-H]^-$ of 8-hydroxy-aristolochic acid (AAIa) (for structure see Figure 1). Positive MALDI-TOF-TOF detected peaks at *m/z* 328.043 and 327.029, representing the molecular ions $[M-H]^+$ and $[M]^+$ of AAIa, respectively (Fig. 4B). The peaks at *m/z* 283.021 and 311.031, representing ions of AAIa fragments, were also found (Fig. 4B). These results show that the detected metabolite is the demethylation product of AAI, 8-hydroxy-aristolochic acid (AAIa) (for structure see Figure 1), which was found to be a detoxication metabolite of AAI (Shibutani et al., 2010). In contrast to hepatic microsomes of WT, WT-BaP and HRN-BaP mice, microsomes of HRN mice did not oxidize AAI (Figs. 3C and 5A). Pretreatment of mice with BaP, a potent CYP1A1/2 inducer (Arlt et al., 2008) greatly stimulated AAI demethylation to AAIa. Even hepatic microsomes of HRN mice treated with BaP were capable of demethylating AAI (Fig. 5A). These results correlated well with the protein expression of Cyp1a1/2 and the EROD activity in the hepatic microsomes (Arlt et al., 2008; compare Fig. 5B).

In contrast to mouse hepatic microsomes, renal microsomes from WT and HRN mice did not oxidize AAI to AAIa under the same experimental conditions (data not shown).

The efficiency of hepatic microsomes from WT mice to form AAIa was compared to that of human, rat and rabbit hepatic microsomes (Supplementary fig. S1). Hepatic microsomes from all these species oxidized AAI to AAIa. Whereas human and rat hepatic microsomes oxidized AAI with similar efficiency, a 1.3-fold lower oxidation of AAI was found in mouse hepatic microsomes compared to human microsomes and even lower activity in microsomes of rabbits (Supplementary fig. S1).

The capacity of different CYPs to demethylate AAI to AAIa was studied using hepatic microsomes of rats treated with CYP inducers. As shown in Figure 6A, hepatic microsomes of rats treated with Sudan I (which induces CYP1A) and PB (which induces CYP2B and -2C) were 1.3 and 1.1 times more efficient to oxidize AAI to AAIa than uninduced microsomes. In contrast, other CYP inducers such as ethanol (which induces CYP2E1) and PCN (which induces CYP3A) decreased AAIa formation. Collectively, these findings strongly suggest that CYP1A1/2 enzymes are predominantly responsible for AAI demethylation to AAIa in mouse and rat liver microsomes.

Oxidation of AAI to AAIa by rat recombinant CYPs in Supersomes™

Further experiments were conducted using microsomes of Baculovirus transfected insect cells (Supersomes™) containing recombinantly expressed rat CYPs, POR and/or cytochrome b_5 (Fig. 6B). Only rat CYPs could be utilized as Supersomes™ containing individual mouse Cyps are not available. Rat CYP1A2 was the most efficient enzyme at demethylating AAI to AAIa, followed by CYP1A1 (Fig. 6B). Rat recombinant CYP2C enzymes were also capable of demethylating AAI, but to a lesser extent (Fig. 6B). No AAIa formation occurred in control incubations with Supersomes™ containing POR alone.

Hepatic microsomes of WT, WT-BaP and HRN-BaP but not of HRN mice activate AAI to species forming DNA adducts

In order to evaluate the role of Por-dependent Cyp-mediated activation of AAI in DNA adduct formation, we carried out *in-vitro* incubations using the same hepatic mouse microsomes as those used in the experiments above, except that incubations were performed under hypoxic conditions. Incubation mixtures were purged with a stream of argon for 3 min before the addition of AAI. Although most of the oxygen was removed, we cannot exclude its presence in the membranes and lumen of microsomes present in the mixtures. AAI was activated by hepatic microsomes of WT, WT-BaP and HRN-BaP mice, generating the same cluster of 3 DNA adducts as those obtained *in vivo* in mice (spots 1, 2 and 3 shown in Figure 1), identified to be dG-AAI (spot 1), dA-AAI (spot 2) and dA-AAII (spot 3). Pretreatment of mice with BaP increased the levels of AAI-DNA adducts catalyzed by hepatic microsomes of both WT and HRN mice (Figs. 5C and 7C and Supplementary Table S2). Because this increase corresponded to an increase in Cyp1a1/2 protein expression and their enzymatic activities (Arlt *et al.,* 2008; compare Figure 5B), our findings indicate that mouse Cyp1a1/2 participate in the metabolic activation of AAI to form DNA adducts. Control incubations carried out in parallel without microsomes, or without DNA, or without AAI, were free of adduct spots in the region of interest (data not shown). Renal microsomes of both mouse strains (WT and HRN) also activated AAI to form the same AAI-DNA adducts, both with similar efficiencies. However, renal microsomes of WT mice were less effective than hepatic microsomes (Fig. 7A, 7B and Supplementary Tables S2 and S3).

Whereas hepatic microsomes of WT, WT-BaP and HRN-BaP mice activate AAI to species forming DNA adducts, those of HRN mice were not active (Figs. 5C and 7). This finding that again parallels Cyp1a1/2 enzyme activity (EROD) (Fig. 5B) might be caused either by the lack of Por-mediated or Por-dependent-Cyp1a1/2-mediated activation of AAI.

To further investigate the role of Por-dependent-Cyp-mediated DNA adduct formation by AAI in mouse hepatic microsomes, inhibitors of several Cyps and Por were utilized. In hepatic microsomes AAI-DNA adduct formation was inhibited with α-NF (which inhibits Cyp1a1/2),

ellipticine (which inhibits Cyp1a1) and furafylline (which inhibits Cyp1a2), but not with ketoconazole (which inhibits Cyp3a) and α-lipoic acid (which inhibits Por) (Fig. 7A and 7C and Supplementary Table S4). These results corroborate the suggestion that Cyp1a enzymes play an important role in AAI-DNA adduct formation in mouse liver, whereas participation of Por or other Cyps such as Cyp3a is negligible in this process. The major role of mouse hepatic microsomal Cyp1a1/2 in AAI activation is in accordance with former findings showing participation of these enzymes in this process in human and rat hepatic microsomes (Stiborová et al., 2001b; 2005a; 2005c).

In contrast to hepatic microsomes, AAI-DNA adduct formation catalyzed by renal microsomes was inhibited by ketoconazole and α-lipoic acid, but not by α-NF, ellipticine or furafylline (Fig. 7B and Supplementary Table S5). An increase in AAI-DNA adduct levels was even produced by α-NF, the compound known to stimulate oxidation of several substrates catalyzed by Cyps of the 3a subfamily (Rendic and DiCarlo, 1997; Ueng et al., 1997; Bořek-Dohalská et al., 2001; Bořek-Dohalská and Stiborová, 2010). Moreover, α-NF was also found to stimulate reduction of some substrates by POR including AAI, thereby increasing levels of AAI-DNA adducts (Stiborová et al., 2001b; 2005a; Hodek et al., 2009). Therefore, these results indicate that Cyp3a and Por might participate in AAI-DNA adduct formation in mouse kidney.

Whereas POR was found to catalyze AAI-DNA adduct formation in human kidney microsomes (Stiborová et al., 2005a), human CYP3A4, the most abundant CYP enzyme in human livers (Rendic and diCarlo, 1997), was inactive (Stiborová et al., 2001b; 2005a; 2005c). Human recombinant CYP3A5, the enzyme that is expressed in human kidney, was tested in this work to investigate its potential to activate AAI. As shown in Supplementary figure 2 and Supplementary Table S6, CYP3A5 expressed in Supersomes^{TM} increases levels of AAI-derived DNA adducts when incubated with DNA *in vitro*, indicating its role in the activation process. This conclusion was also supported by the inhibition of AAI-DNA adduct formation in this system by ketoconazole.

Metabolic activation of AAI mediated by mouse hepatic and renal cytosols in vitro

In initial experiments, we investigated the efficiency of hepatic and renal cytosolic samples of WT and HRN mice to generate adduct forming species from AAI. Incubations were carried out in the presence of NADPH, a cofactor of Nqo1, the enzyme shown to be most efficient in AAI activation (Stiborová et al., 2002a; 2003a; 2011). Similarly to human hepatic and renal cytosols (Stiborová et al., 2003a; 2011), cytosols of both organs and of both mouse strains were capable of activating AAI to form DNA adducts (see Fig. 8A and Supplementary Table S7 for hepatic cytosols and Supplementary Table S8 for renal cytosols), but mouse renal cytosols were less effective than hepatic cytosols (Supplementary Tables S7 and S8). Interestingly, hepatic cytosolic samples of HRN mice were more than 3-fold more efficient in activating AAI to DNA adducts than those of WT mice (Fig. 8A and Supplementary Table S7).

In hepatic cytosols, we also tested whether pretreatment of WT and HRN mice with BaP, a Nqo1 inducer (Elovaara et al., 2007; Hockley et al., 2007; Vondráček et al., 2009), influences the formation of AAI-DNA adducts. Indeed, more than 3- and 6-fold higher levels of AAI-DNA adducts catalyzed by hepatic cytosols of HRN-BaP and WT-BaP mice than untreated mice were determined, respectively (Fig. 8A and Supplementary Table S7). This was paralleled by an increase in Nqo1 protein expression and enzyme activity in these samples (Fig. 8B and 8C). These results demonstrate that Nqo1 plays an important role in livers of both WT and HRN mice, analogous to its role in human and rat liver cytosols (Stiborová et al.., 2002a; 2003a; 2011). Indeed, participation of Nqo1 in AAI-DNA adduct formation with mouse hepatic cytosols was confirmed by the effect of dicoumarol, an Nqo1 inhibitor, in reducing DNA binding (Supplementary fig. 3 and Supplementary Table S9).

DISCUSSION

A large body of evidence indicates that metabolic activation and detoxication of AAI influences its nephrotoxic and carcinogenic effects. Demethylation of AAI to AAIa is believed to be detoxication reaction, because AAIa was found to be much less toxic than AAI (Shibutani et al., 2010). In addition, AAIa or its conjugates, the glucuronide, the acetate and the sulfate esters, are readily excreted in urine (Chan et al., 2006; 2007).

Formation of AAIa was recently found to be catalyzed mainly by human CYP1A1 and -1A2 *in vitro* (Šístková et al., 2008; Rosenquist et al., 2010) and by mouse Cyp1a2 *in vivo* (Rosenquist et al., 2010). However, CYP1A1 and -1A2 are the enzymes that also activate AAI (Stiborová et al., 2001b; 2005a; 2005c). We therefore investigated participation of these and other CYPs both in detoxication and in activation of AAI in detail.

HRN mice carry a deletion of the *Por* gene in hepatocytes (Henderson and Wolf, 2003; Henderson et al., 2003b), and thus lack Cyp activity in the liver. AAI-DNA adducts were formed in all organs of both WT and HRN mice treated with AAI. These findings suggest that AAI is distributed *via* the blood stream to other organs and that these tissues have the metabolic capacity to reductively activate this carcinogen. Moreover, our results demonstrate that Por-dependent, Cyp-mediated metabolism of AAI in the liver dictates the formation of DNA adducts by this compound also in extrahepatic organs in mice *in vivo*. Besides such an *in vivo* study, we performed *in-vitro* experiments utilizing subcellular systems (*i.e.* microsomes and cytosols) of the liver, the organ that is predominantly responsible for the biotransformation of many xenobiotics, as well as the kidney, the target organ for AAI toxicity. We demonstrated that hepatic Cyps oxidize AAI, thereby decreasing its concentrations in liver of WT mice, and lower amounts of AAI are distributed to extra-hepatic organs than in HRN mice. Indeed, lower levels of AAI-DNA adducts were found in tissues of WT mice relative to HRN mice. Absence of AAI demethylation activity (*i.e.* detoxication) in livers of HRN mice was also shown by *in vitro* experiments. Whereas hepatic microsomes isolated from WT mice oxidized AAI to AAIa, those from HRN mice were not capable

of catalyzing this reaction. These results fit well with the proposed scheme of AAI metabolism (see Fig. 1). If AAI is not oxidized to AAIa in the liver, it is activated by several enzymes with nitroreductase activity (for a review, see Stiborová et al., 2008b; 2008c) to form a cyclic acylnitrenium ion generating DNA adducts (Fig. 1). Our results are in accordance with another study showing that mice that lack hepatic Por-dependent Cyp activity (also HRN mice, but a different strain) are extremely sensitive to AA toxicity (Xiao et al., 2008). Together with the present study, these results demonstrate that the HRN mouse model used in the study is an excellent model not only to further investigate the toxic effects of AAI, but also of other toxic agents.

We found that the hepatic CYP enzymes of the 1A subfamily of mice and rats are crucial for *in vitro d*emethylation of AAI in rodents, similar to its role in human systems (Šístková et al., 2008; Rosenquist et al., 2010). Inducers of these enzymes such as BaP (Arlt et al., 2008) and Sudan I (Stiborová et al., 2002b) increased demethylation of AAI catalyzed by mouse and rat hepatic microsomes. The results found in *in-vitro* experiments using mouse hepatic microsomes correspond well to the situation *in vivo*; as shown by Rosenquist and co-workers (2010), treatment of mice lacking Cyp1a2 protein expression (Cyp1a2 knockout mice) with AAI led to elevated levels of AAI-DNA adducts in the renal cortex and to an increase in microalbuminuria, an indicator of renal tubule dysfunction, relative to WT mice. A major role of CYP1A in AAI demethylation was also confirmed in the present work by utilizing rat recombinant CYP enzymes; rat CYP1A2, followed by CYP1A1, were the most efficient in AAI demethylation to AAIa. In addition to rat CYP1A1/2, rat CYP enzymes of the 2C subfamily were also capable of demethylating AAI, but with much lower efficiencies than CYP1A. But because CYP2C enzymes in rat hepatic microsomes constitute 55% of all CYP, while CYP1A only 2% (Nedelcheva and Gut, 1994; Rolsted and Kissmeyer, 2008), the contributions of CYP2C enzymes to AAI demethylation in rat livers might be important.

The increase in AAI-DNA adduct formation found in HRN mice is the combined result of the lack of AAI detoxication by demethylation catalyzed by hepatic Cyps, and the reductive activation

of AAI by Nqo1, the most effective activating enzyme in human and rat liver and kidney (Stiborová et al., 2002a; 2003a; 2011). Expression and activity of this enzyme in the liver of HRN mice were higher than in the liver of WT mice. This higher Nqo1 activity results in better activation of AAI to DNA adduct formation with hepatic cytosol of HRN mice. Likewise, increased AAI-DNA adduct formation was found with renal cytosol of HRN mice as compared with WT mice. Of note, it was reported that polymorphisms in the human NQO1 gene are important in AAI-induced nephropathy [i.e. BEN, a disease that is associated with dietary exposure to AA (Arlt et al., 2007; Grollman et al., 2007)] and/or carcinogenicity (Toncheva et al., 2004; Atanasova et al., 2005). Indeed, one of the NQO1 polymorphisms, the genotype NQO1*2/*2, was shown to predispose patients suffering from BEN to develop urothelial cancer (OR=13.75, 95%CI 1.17-166.21) (Toncheva et al., 2004). This finding together with the demonstration of the importance of NQO1 in AAI activation could be an explanation for cancer induction by AAI in only some of the AAN and BEN patients. The results found in this and former (Stiborová et al., 2002a; 2003a; 2011) studies strongly support the hypothesis (Stiborová et al., 2008c) that a key point determining the carcinogenic and nephrotoxic effects of AAI lies in the balance of activities of reductases such as NQO1, catalyzing AAI-DNA adduct formation, and enzymes such as CYPs, which detoxify AAI to AAIa.

It should be emphasized however that under the hypoxic (anaerobic) conditions, mouse hepatic Cyp1a enzymes were also capable of reducing AAI to adduct forming species *in vitro*. Whereas hepatic microsomes of WT mice catalyzed activation of AAI to form AAI-DNA adducts, only hepatic microsomes of HRN mice pretreated with BaP, an inducer of Cyp1a, were able to catalyze this reaction, but not microsomes from untreated HRN mice. The major role of Cyp1a in AAI-DNA adduct formation by hepatic microsomes was also demonstrated using selective inhibitors. Inhibitors of Cyp1a1 and -1a2, but not of Por and Cyp3a, decreased levels of AAI-DNA adducts formed by hepatic microsomes. These findings demonstrate that besides the levels of Cyp1a

expression in the liver, the *in vivo* oxygen concentration in tissues will affect the balance between nitroreduction and demethylation of AAI, thereby influencing its toxicity and carcinogenicity. Taking into account all available data, we propose that the pathways of AAI metabolism are mainly dictated by the binding affinities of AAI to CYP1A or NQO1, and their enzymatic turnover as well as by the balance between the efficiency of CYP1A to oxidize and reduce AAI.

In contrast to mouse hepatic microsomes, Por and Cyp3a enzymes seem to be more important in AAI-DNA adduct formation in renal microsomes. Previously POR was found to be an efficient enzyme catalyzing activation of AAI in human kidney (Stiborová et al., 2005a). However, the participation of renal mouse Cyp3a in AAI-DNA adduct formation was initially rather surprising, because human CYP3A4 that was tested previously for its efficiency to activate AAI was found to be ineffective (Stiborová et al., 2005a). CYP3A5, another human enzyme of the CYP3A subfamily, predominantly expressed in human kidney, was herein found to activate AAI. This finding might explain the results of a pilot genetic study (Atanasova et al., 2005), which found a weak association between human CYP3A5*1 allele and BEN.

In conclusion, although the impact of individual metabolic enzymes on the nephrotoxicity and carcinogenicity of AAI is still not entirely resolved, one question was unambiguously answered by our present work and other recent studies (Xiao et al., 2008, Rosenquist et al. 2010), namely that hepatic Cyp1a enzymes detoxify AAI in mice, thus decreasing its renal toxicity. The evaluation of inter-individual variations in the human enzymes that play a major role in AAI activation and detoxication, including their genetic polymorphisms, remain a major challenge to explain human individual susceptibility to AAI, and to predict the risk of cancer among the AAN and BEN patients.

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Legends for Figures

FIG. 1**.** Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; NR, nitroreduction; UGT, UDP glucuronyl transferase; SULT, sulfotransferase. Inset: Autoradiographic profile of AA-DNA adducts in kidney of HRN mice treated with 50 mg/kg bw of AAI by using the nuclease P1 enrichment version of the ^{32}P-postlabeling assay. The origin, in the bottom left-hand corner, was cut off before exposure. Spot 1, dG-AAI; spot 2, dA-AAI; spot 3, 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII).

FIG. 2**.** Quantitative TLC ^{32}P-postlabeling analysis of DNA adducts formed by AAI in organs of HRN and WT mice treated orally with 10 (A) or 50 mg AAI/kg bw for 24 hours (B). F, fold increase in DNA adducts in HRN mice compared with WT mice. RAL, relative adduct labeling. ND, not detected. Values are given as means ± SD (n = 3). Values significantly different from WT mice: *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test).

FIG. 3**.** HPLC chromatograph of AAIa metabolite (peak r.t. at 28.3 min) and AAI (peak r.t. at 36 min) produced by hepatic microsomes of WT mice incubated with AAI and NADPH (A), by hepatic microsomes of WT mice incubated with AAI without NADPH (B) and by hepatic microsomes of HRN mice incubated with AAI and NADPH (C). The peaks with the characterized AAI metabolite (AAIa) and the parent AAI are indicated in the chromatograms.

FIG. 4**.** Identification of AAI metabolite as AAIa. Negative NALDI-TOF/TOF (A) and positive MALDI-TOF/TOF (B) of AAIa. The peaks at *m/z* 348.736 and 366.765 in panel A are matrix peaks.

FIG. 5**.** Oxidation of AAI to AAIa by murine hepatic microsomes isolated from livers of HRN and WT mice, control mice (untreated) or mice pretreated (i.p.) with 125 mg BaP/kg bw daily for 5 days (Arlt et al., 2008) (A); Cyp1a enzymatic activity (EROD activity) in hepatic microsomes (B). DNA adduct formation by AAI activated with hepatic microsomes as determined by TLC ^{32}P-postlabeling

(C). RAL, relative adduct labeling. ND, not detected. All values are given as means ± SD (n = 3). Values significantly different from control (untreated) WT mice: ***$p<0.001$ (Student's t-test).

FIG. 6**.** Oxidation of AAI by rat hepatic microsomes (A) or recombinant CYPs of rat (B). Values are given as means ± SD (n = 3). Values significantly different from hepatic microsomes of control (untreated) rats: *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test). Sudan I, phenobarbital (PB), ethanol (EtOH) and pregnenolone-16α-carbonitrile (PCN) were used as inducers of rat CYPs. 1 mg microsomal protein or 50 μM rat recombinant CYPs and 10 μM AAI were used in incubations (see Materials and Methods). Control, control Supersomes™ containing POR alone (50 nM). ND, not detected.

FIG. 7**.** Effect of different inhibitors on DNA adduct formation by AAI activated with hepatic or renal microsomes isolated from HRN and WT mice as determined by TLC ^{32}P-postlabeling. Hepatic (A) and renal microsomes of control mice (untreated) (B). (C) Hepatic microsomes of mice pretreated (i.p) with 125 mg/kg bw BaP daily for 5 days (Arlt et al., 2008). Inhibitors α-NF, α-naphthoflavone; E, ellipticine; FF, furafylline; KC, ketoconazole; α-LA, lipoic acid were used in the experiments. F, fold increase in DNA binding in hepatic or renal microsomes from WT mice compared with HRN mice. RAL, relative adduct labeling. Control, without cofactor. ND, not detected. Values are given as means ± SD (n = 3). Values significantly different from incubations without inhibitors: ***$p<0.001$ (Student's t-test).

FIG. 8**.** DNA adduct formation by AAI activated with hepatic cytosols isolated from livers of HRN and WT mice, control mice (untreated) or mice pretreated (i.p.) with 125 mg/kg bw BaP daily for 5 days (Arlt et al., 2008) as determined by TLC ^{32}P-postlabeling (A). RAL, relative adduct labeling. Nqo1 protein expression in hepatic cytosols as determined by Western blotting (see insert) (B). Nqo1 enzymatic activity in hepatic cytosols (C). Human recombinant NQO1 (Sigma; NQO1 stan, see insert B) was used to identify the Nqo1 band in murine cytosols. All values are given as means

± SD (n = 3). Values significantly different from control (untreated) mice: ***$p<0.001$ (Student's t-test).

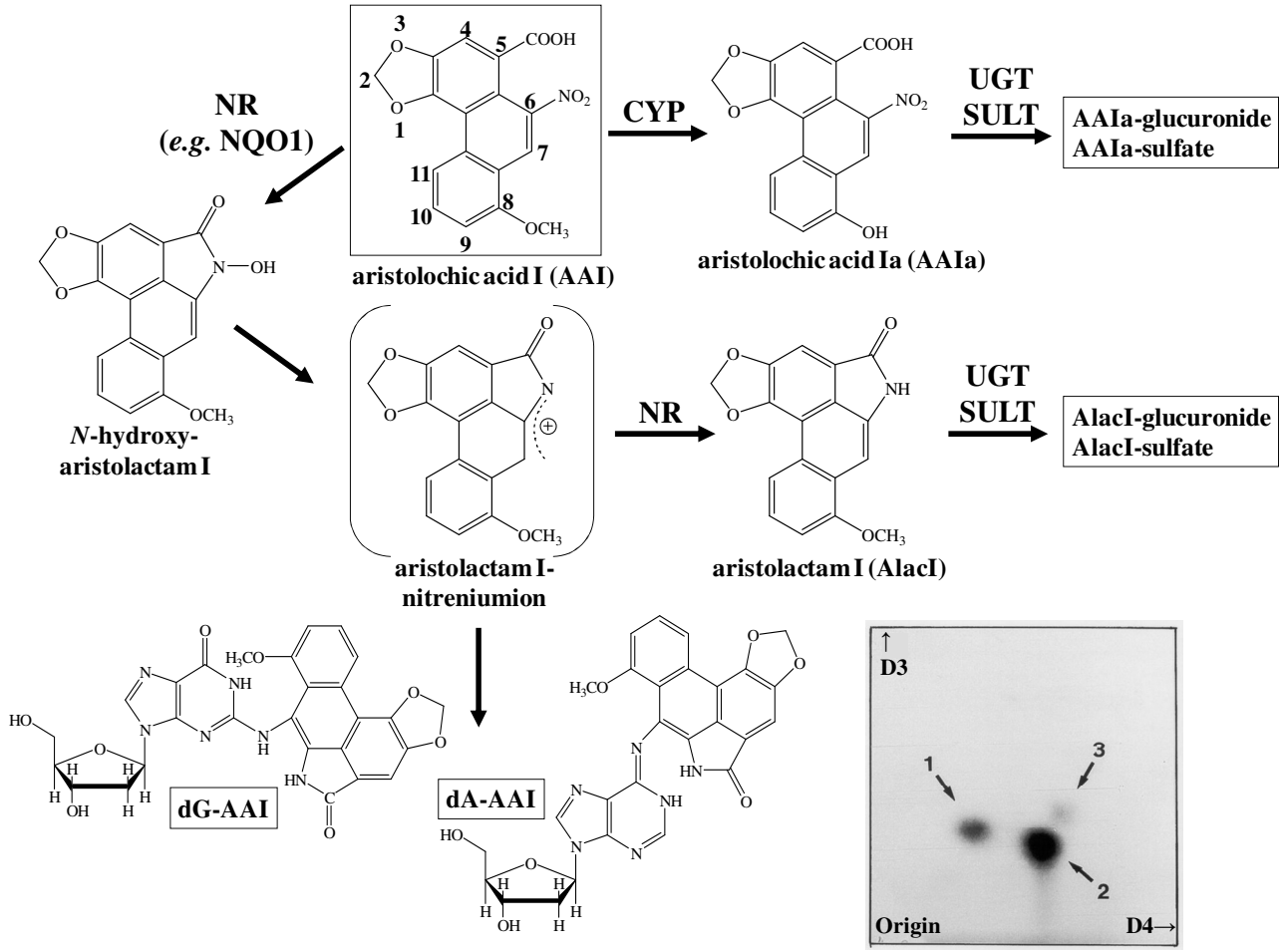
NR
(e.g. NQO1)
aristolochic acid I (AAI)
CYP
aristolochic acid Ia (AAIa)
UGT
SULT
AAIa-glucuronide
AAIa-sulfate
N-hydroxy-
aristolactam I
aristolactam I-
nitreniumion
NR
aristolactam I (AlacI)
UGT
SULT
AlacI-glucuronide
AlacI-sulfate
dG-AAI
dA-AAI
D3
D4→
Origin

Figure 1

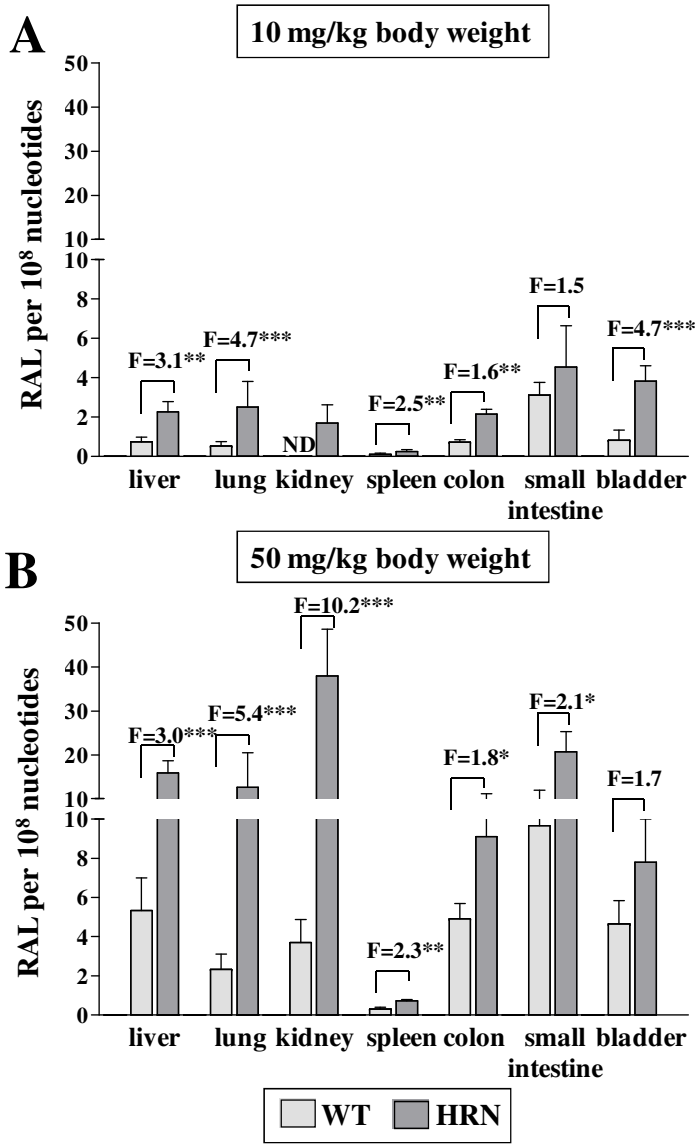
A
10 mg/kg body weight
RAL per 10^8 nucleotides
F=3.1**
F=4.7***
ND
F=2.5**
F=1.6**
F=1.5
F=4.7***
liver
lung
kidney
spleen
colon
small intestine
bladder
B
50 mg/kg body weight
RAL per 10^8 nucleotides
F=3.0***
F=5.4***
F=10.2***
F=2.3**
F=1.8*
F=2.1*
F=1.7
liver
lung
kidney
spleen
colon
small intestine
bladder
WT
HRN

Figure 2

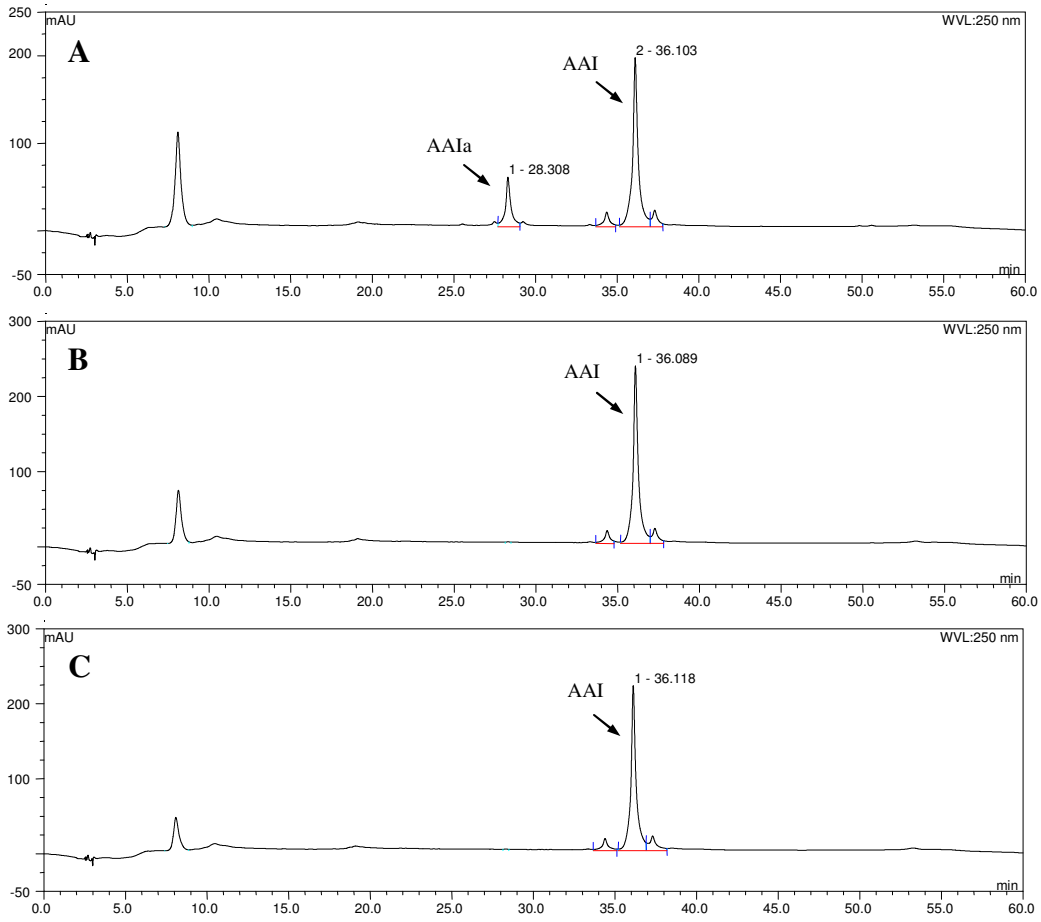
A
mAU
WVL:250 nm
AAI
2 - 36.103
AAIa
1 - 28.308
min
B
mAU
WVL:250 nm
AAI
1 - 36.089
min
C
mAU
WVL:250 nm
AAI
1 - 36.118
min

Figure 3

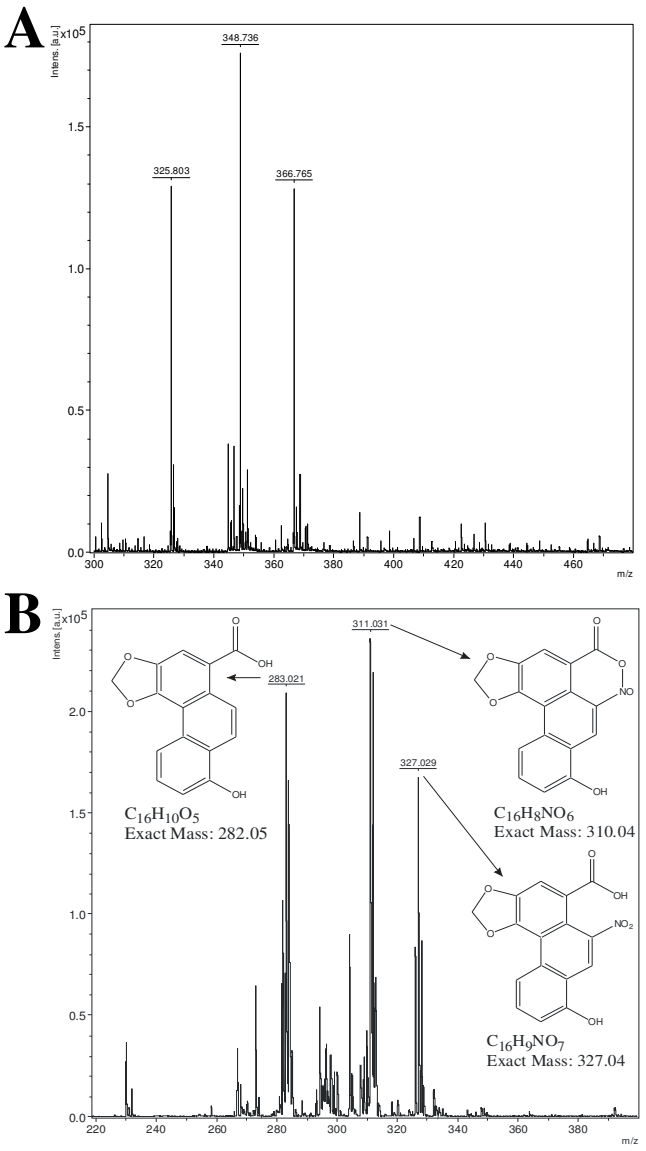
A
Intens. [a.u.]
x10^5
348,736
325,803
366,765
m/z
B
311,031
283,021
327,029
C16H10O5
Exact Mass: 282.05
C16H8NO6
Exact Mass: 310.04
C16H9NO7
Exact Mass: 327.04
m/z

Figure 4

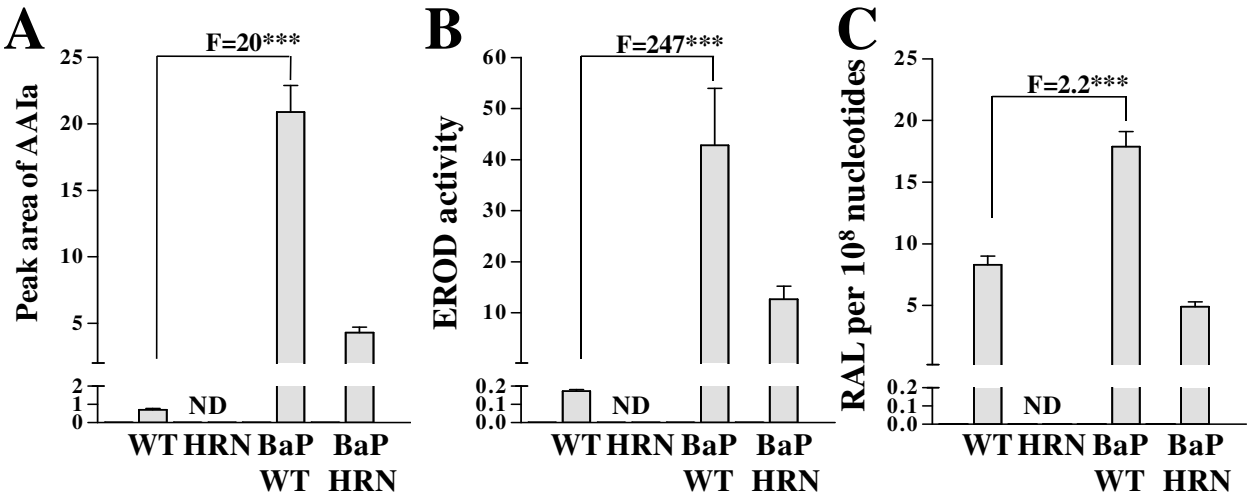
A
Peak area of AAIa
F=20***
25
20
15
10
5
2
1
0
ND
WT
HRN
BaP WT
BaP HRN
B
EROD activity
F=247***
60
50
40
30
20
10
0.2
0.1
0.0
ND
WT
HRN
BaP WT
BaP HRN
C
RAL per 10^8 nucleotides
F=2.2***
25
20
15
10
5
0.2
0.1
0.0
ND
WT
HRN
BaP WT
BaP HRN

Figure 5

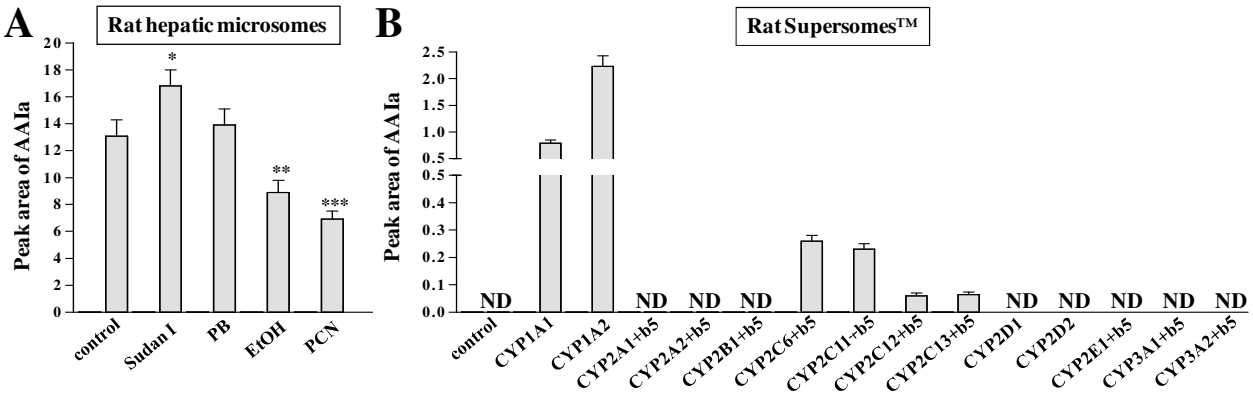
A
Rat hepatic microsomes
Peak area of AAIa
control
Sudan I
PB
EtOH
PCN
B
Rat Supersomes™
Peak area of AAIa
ND
control
CYP1A1
CYP1A2
CYP2A1+b5
CYP2A2+b5
CYP2B1+b5
CYP2C6+b5
CYP2C11+b5
CYP2C12+b5
CYP2C13+b5
CYP2D1
CYP2D2
CYP2E1+b5
CYP3A1+b5
CYP3A2+b5

Figure 6

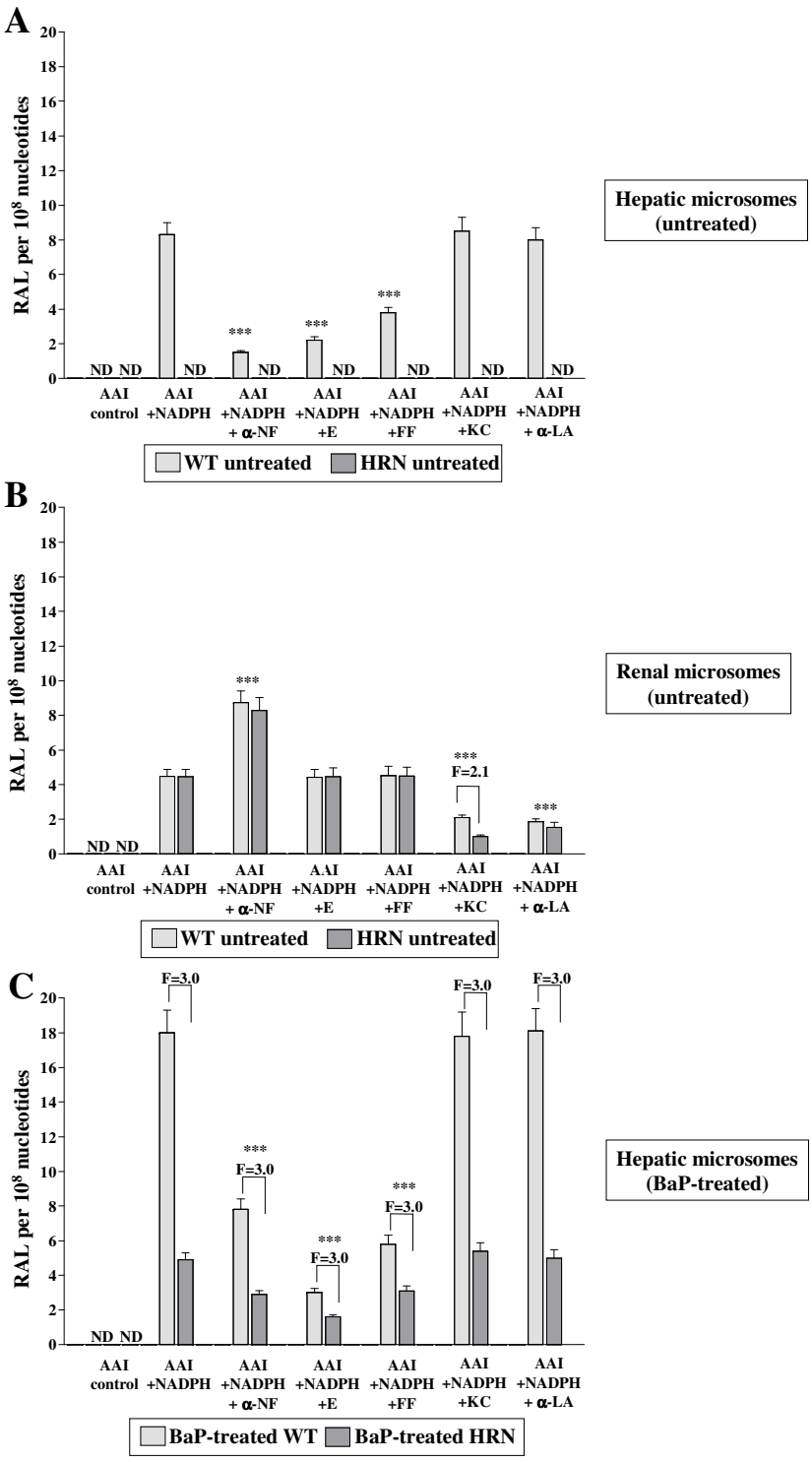
A
RAL per 10^8 nucleotides
Hepatic microsomes (untreated)
WT untreated
HRN untreated
B
RAL per 10^8 nucleotides
Renal microsomes (untreated)
F=2.1
WT untreated
HRN untreated
C
RAL per 10^8 nucleotides
Hepatic microsomes (BaP-treated)
F=3.0
BaP-treated WT
BaP-treated HRN
ND
***
AAI control
AAI +NADPH
AAI +NADPH + α-NF
AAI +NADPH +E
AAI +NADPH +FF
AAI +NADPH +KC
AAI +NADPH + α-LA

Figure 7

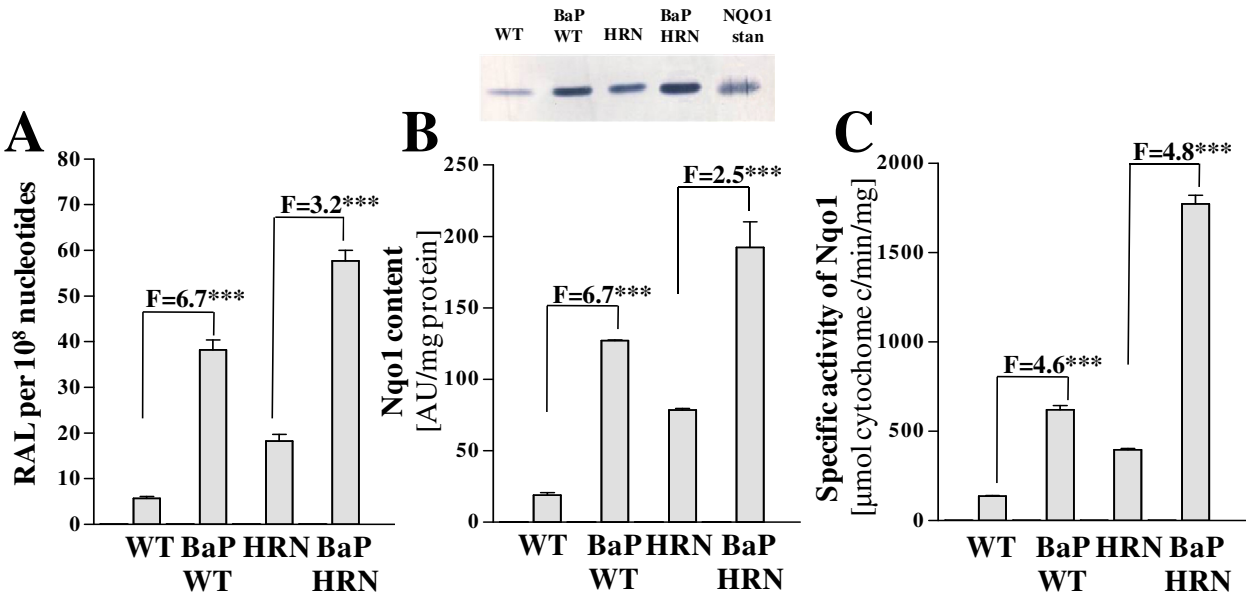
A
RAL per 10^8 nucleotides
F=6.7***
F=3.2***
WT
BaP WT
HRN
BaP HRN
B
WT
BaP WT
HRN
BaP HRN
NQO1 stan
Nqo1 content
[AU/mg protein]
F=6.7***
F=2.5***
C
Specific activity of Nqo1
[µmol cytochome c/min/mg]
F=4.6***
F=4.8***

Figure 8